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Genotoxic effects of combined multiple stressors on *Gammarus locusta* haemocytes: Interactions between temperature, pCO_2 and the synthetic progestin levonorgestrel☆

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ABSTRACT

Climate change and pharmaceutical contamination are two priority research topics due to their impacts in the aquatic ecosystems and in the food chain structure. In the bottom of many food chains are the invertebrates, like the amphipods, which are important environmental and ecotoxicological models. In this study, we combined the increase of temperature [ambient and warming temperature], pCO_2 [normocapnia and hypercapnia] and the synthetic progestin levonorgestrel (LNG) [environmentally relevant concentration (10 ng L^{-1}) and 100-fold higher (1000 ng L^{-1})] to evaluate the genotoxic effects on the amphipod *Gammarus locusta* haemocytes, using the comet assay technique. Additionally, the study examined protective/potentiating effects of the three tested factors against hydrogen peroxide (H_2O_2)-induced DNA damage in haemocytes after *ex vivo* exposure. Our data revealed no significant effects of any of the three stressors on DNA damage of *G. locusta* haemocytes or protection against H_2O_2-induced DNA damage after twenty-one days exposure. Only a significant effect of the solvent was visible, since it was able to induce higher DNA damage (i.e. strand breaks) on exposed individuals. On the other hand, LNG exposure seemed to induce a slight increase of DNA damage after H_2O_2 exposure. Our findings suggest that more short-term studies to conclude about the genotoxicity and/or protective effects of the stress factors in *G. locusta* should be made, attending to the fast turnover rate of repairing cells that could have masked impacts seen only after the end of the experiment.

1. Introduction

Anthropogenic activities and population increase are the main drivers for greenhouse gases production, resulting into the rise of carbon dioxide (CO_2) levels. Only in the past 10 years, CO_2 levels increased 22 ppm, reaching now levels of 408.30 ppm (McGee, 2018). As a consequence, oceans are becoming warmer and more acidic as atmospheric concentrations of CO_2 increase (IPCC, 2014). Under hypercapnic and warmer conditions, negative pressures were saw on the calcification, energy metabolism, reproduction, and in development/growth of many marine calcifiers (Byrne and Przeslawski, 2013); weaker shell/exoskeletons can also lead to higher mortality rates and lower soft tissue growth (Goncalves et al., 2017), as well as impaired immune functions (e.g. increase in apoptosis and reactive oxygen species production; Wang et al., 2016).

As serious as global warming is the issue of chemical pollution. Environmental endocrine disrupting chemicals (EDCs) are ubiquitous and can affect reproductive, neurological and immune pathways of different trophic level organisms (Segner et al., 2003). Among the most potent EDCs are sex-steroid hormones (Fent, 2015 and references therein). In particular, progestins (also known as

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gestagens) are primarily produced as contraceptive pharmaceuticals and they can also be part of hormone replacement therapies, prevention of endometrial cancer, among others (Fent, 2015; Lopez de Alda et al., 2002). Regarding the synthetic progestin levonorgestrel (LNG) – known as a contraceptive and a post-coital contraception pill – studies already addressed for the presence of this progestin in surface waters even after their treatment, ranging from 0.2 up to 30 ng L^{-1} (Fick et al., 2010; Kumar et al., 2015; Mira et al., 2009; Viglino et al., 2008).

Due to its presence in the aquatic ecosystems, questions regarding how the LNG can act as an EDC and affect aquatic organisms at trace concentrations (ng L^{-1}) have been raised and, as mentioned by Kumar et al. (2015), few experiments conducted mainly in fish (at different growth stages) demonstrated decrease egg production, inhibition in the onset of spermatogenesis and decrease of 11-ketotestosterone in both sexes. Studies in bivalves also reported disrupting effects of that progestin (Contardo-Jara et al., 2011; Dimastrogiovanni et al., 2015), however invertebrates are still poorly explored.

As far as we know, the combined effects of EDCs, particularly progestins and climate change on DNA stability in aquatic organisms, like crustaceans is unknown. Genotoxic agents may ripple a cascade of adverse alterations from cellular to individual level affecting survival, reproduction and development (Gagné and Gagné, 2014; Sukumaran and Grant, 2013). A very quick, sensitive, and relatively inexpensive method, to detect DNA damage is the single-cell gel electrophoresis assay — also called "Comet assay" —where only a small number of cells are needed providing the opportunity to study DNA damage at the level of individual cells (Collins et al., 2014).

Due to their ecological niche importance, amphipods are broadly used as a reliable bioindicator of contamination and environmental changes (Castro et al., 2006; Costa et al., 2005; De Lange et al., 2006; Lacaze et al., 2011a, 2011b, 2010; Maltby et al., 2009; Neuparth et al., 2005, 2002). As a good ecotoxicological model, amphipods have a strong ecological relevance and high sensitivity to contaminants, which associated to the short-life cycle and ease maintenance and breed, makes it a good model to assess the effects of contaminants along its life cycle (Cardoso et al., 2018; Jacobson et al., 2008).

Due to the gap of information about the effects of climate change and environmental pollution interactions on aquatic species, we aimed to explore the genotoxic effects of combined multiple stressors (temperature, pCO_2, and LNG concentrations) on the species *Gammarus locusta*, using comet assay as a genotoxicity tool. Besides the genotoxic effect (strand breaks – SBs – including alkali-labile sites), we also assessed the capacity to stand against hydrogen peroxide (H_2O_2)-induced DNA damage in haemocytes after *ex vivo* exposure.

2. Material and Methods

2.1. Animals origin and maintenance

Sub-adult animals (with approximately 3–4 weeks), descendent from a permanent laboratory culture at CIIMAR (Portugal) were selected for this trial. Prior to the experiment, animals were acclimatized for 7 days with a summer photoperiod set to 18 h light: 6 h dark, salinity 33–35 in *ad libitum* feed basis. The bottom of the tanks was covered with sand (1 cm) and pebbles to mimic the original conditions; sand and pebbles were previously burned for 8 h at 450 °C to avoid any external contamination, as described by Cardoso et al. (2013). Water was fully changed twice a week. All abiotic conditions (pH, temperature, photoperiod and dissolved oxygen) were similar to the Sado River estuary (Portugal), which was the original local of the *G. locusta* population. Physico-chemical parameters, such as ammonia (0.43 ± 0.15 mg L^{-1}) and nitrites (0.10 ± 0.10 mg L^{-1}) averaged acceptable amounts for a maintenance system.

2.2. Experimental design

The experimental set-up followed a full factorial design as described by Cardoso et al. (2018). Briefly, stress factors such as temperature [ambient (18 °C) and warming (22 °C)], pCO_2 [normocapnia (400 μatm ≈ pH 8.1) and hypercapnia (1600 μatm ≈ pH 7.6)], and levonorgestrel [an environmental concentration (10 ng L^{-1}) and a supra-environmental concentration, 100-fold higher (1000 ng L^{-1})], plus the control and the solvent control (ethanol 0.01%) were settled making a total of 16 treatments; each treatment with 6 replicates (glass cups of 650 ml each). Exposure to LNG was done three times a day, to simulate episodic environmental discharges. The experiment run for twenty-one days. Salinity and photoperiod were similar to the acclimation period for all the treatments. Individuals, belonging to the warming condition (22 °C) were subject to a gradual increase of temperature of 1 °C day^{-1}. The continuous flow-through system was composed by 4 levels as the model suggested by Cornwall and Hurd (2016; more details in Fig. 1).

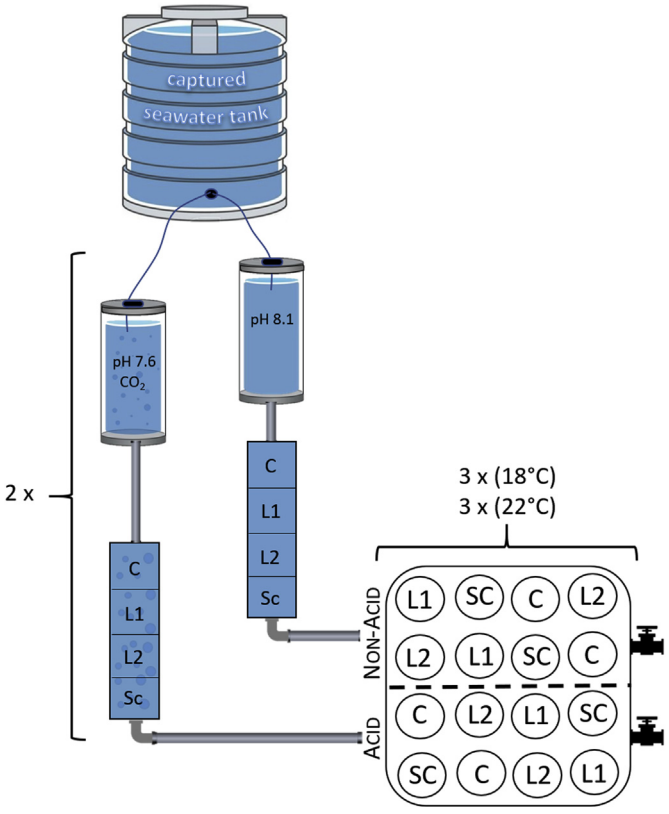

Fig. 1. Schematic representation of the experimental set-up, with four different levels: I – single seawater tank (350 L, seawater collected from Perafita, Portugal (41.224088, −8.717479)), II – four CO_2 mixing tanks (acidified and non-acidified 50 L each), III – sixteen contaminant mixing flasks (5 L each); IV – ninety-six experimental units (0.55 L each), randomly distributed by the 6 water baths, using the random sequence generator (random.org); C – control; L1 – LNG 10 ng L^{-1}; L2 – LNG 1000 ng L^{-1}; SC – solvent control.

2.3. System maintenance

Prior to its use, water quality was guaranteed by measuring physico-chemical parameters such as, nitrites, ammonia, salinity, pH, dissolved oxygen, and temperature. Before its use, water also passed through two filters of 25 μm and 100 μm. Water flow (0.009 $Lmin^{-1}$) was maintained by gravity and controlled by a float valve; the same mechanism was employed to control water in CO_2 and in the contaminant mixing tanks. Water acidity was manipulated by diffusing CO_2 from a gas tank, where pH was controlled through a pH stat system (Aqua Medic® AT Control-SW, version 9.0). Both waters (acidified and non-acidified) were then distributed to four tanks each according to the contamination level (*i.e.* control (C), solvent control (SC)), L1 and L2 concentration (10 and 1000 ng L^{-1} of LNG, respectively). Water temperature was maintained by water baths (30 L) and controlled by AT Control-SW. Photoperiod was maintained with artificial light suitable for marine set ups. Aeration on each experimental unit was continuously and individually maintained, using air stones. Contaminated water was collected to an external flask, directed to a contamination tank (20 L) where water was continuously pumped to the decontamination chamber (120 L); there, water was passed through activated-charcoal filter before being eliminated.

For this study three individuals (in a total of 288 animals) were used per replicate which were fed with *Ulva* spp (0.07 g of $ww^{-1} \pm 0.1$, per individual), from a total of 6 flasks, which is the number of replicates ($n = 6$). Algae was renewed three times a week.

2.4. Cell isolation and viability

Animals were not fed the day prior to sampling to avoid crossed contamination and minimize the effect of diet. After twenty-one days exposure, haemolymph of 288 individuals, distributed along 96 experimental units, was collected following the procedure of Weber et al. (2003). Gammarids were individually placed on top of ice cubes until complete immobilization; afterwards, animals were sacrificed. Haemolymph (ca. 10 μL per individual) was sampled by inserting the needle (25 G, 0.5 mm bore size and 16 mm needle length), previously heparinized, between the cephalon and the first periomere, as described by Lacaze et al. (2010). Haemolymph was then, carefully transferred to a 1.5 mL tube placed on ice. Haemocytes viability was assessed microscopically using the trypan blue exclusion test (0.1% in PBS) and cells counted in a Neubauer haemocytometer. Cell identification was carried on through cytocentrifugation (5 min, 1500 rpm), stained after with Diff-Quick and observed at light microscope.

2.5. Comet procedure and analyses

Comet assay technique followed the methodology previously described by Lacaze et al. (2010); Ramos et al. (2010) where some optimizations were done for the *G. locusta* cells. The haemolymph (10 μL) was mixed with 150 μL of low melting point agarose (0.5%) and divided by two precoated slides with 1% normal melting point agarose (70 μL with about 2.0×10^4 cells were placed on each precoated slide) and covered immediately with coverslips. For each haemolymph sample, two slides were performed, one for DNA damage (SBs) evaluation and other to assess effects against H_2O_2-induced DNA damage. After 10 min on ice, the coverslips were removed and the slides used to assess effects against oxidative damage, were treated with H_2O_2 (40 μM) for 5 min on ice, to induce SBs. After, H_2O_2 treated slides were fully immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base, pH 10 plus 1% (v v^{-1}) Triton X-100) for 2 h at 4 °C in the dark. After lysis, slides were rinsed in distilled water and placed in a horizontal electrophoresis chamber with electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 40 min at 4 °C to allow the DNA unwind before electrophoresis for 20 min at 4 °C (1 V cm^{-1} and ~300 mA). Subsequently, slides were washed three times with distilled water for 5 min and then dehydrated two times with 96% ethanol for 5 min. After dried, 20 μl of 4′,6-Diamidino-2-Phenylindole, dihydrochloride (DAPI; 1 μg ml^{-1} in water) was applied to each sample and comets were analysed in a fluorescence microscope (Olympus IX71, Tokyo, Japan) using a 20× objective.

Images were collected and the percentage of tail intensity scored using OpenComet Software (Gyori et al., 2014). At least 100 comets were scored per individual and then a mean of each individual was calculated to determine the mean per treatment. To evaluate the DNA damage distribution, data were organized in classes according to the % of DNA in tail [class 0 (0–5%); class 1 (5–20%); class 2 (20–40%); class 3 (40–75%); class 4 (>75%)] and the frequency of comet in each class determined, as recommended by Azqueta et al. (2009). To evaluate the H_2O_2-induced DNA damage, the basal DNA damage (that corresponds to the % of DNA in tail without H_2O_2 exposure) was subtracted to DNA damage after H_2O_2 exposure, and the results expressed as mean ± SE. Cumulative frequency of nucleoids on class 4 was calculated by addition of the nucleoid frequency on each experimental condition.

2.6. Statistical analysis

Experimental results were examined using linear models (LM). In all the models, significant predictors were selected from the full models by removing sequentially those predictors of higher order and with the higher p-values and comparing the reduced model with the original one using analyses of variance (ANOVA). When significant interactions were found, treatments were compared using p-adjusted Tukey test ($p < 0.05$). Lsmeans package for R was used to perform these tests a posteriori (Lenth, 2016). All the statistical analyses were run in R environment (R Core Team, 2016). Assumptions for the linear models were checked by examining the residual plots. In the case of response variables with Gaussian probability distribution and when required, data were log transformed to reduce heteroscedasticity.

3. Results

3.1. Experimental design and maintenance

During the experiment, water temperature and pH were stable, presenting short 95% confidence intervals and small standard deviations, respectively (ambient temperature - 18.19–18.26 °C ± 0.45, warming temperature - 21.7–21.8 °C ± 0.59, normocapnia - 8.04–8.05 pH ± 0.08 and hypercapnia - 7.59–7.65 pH ± 0.13), indicating a successful design and maintenance of the assay. The survival rate varied among the treatments, ranging from a maximum of approximately 80–90% at 18 °C pH 8.1 to a minimum of 40% for the warming condition (22 °C pH 8.1).

3.2. Cell isolation and viability

A representative sample of haemolymph was analysed in terms of cell viability, which was higher than 80% for all treatments. The obtained cytospin samples were characterized by different haemocyte types as can be seen on Fig. 2.

3.3. Genotoxic effects

DNA damages were estimated only after twenty one days

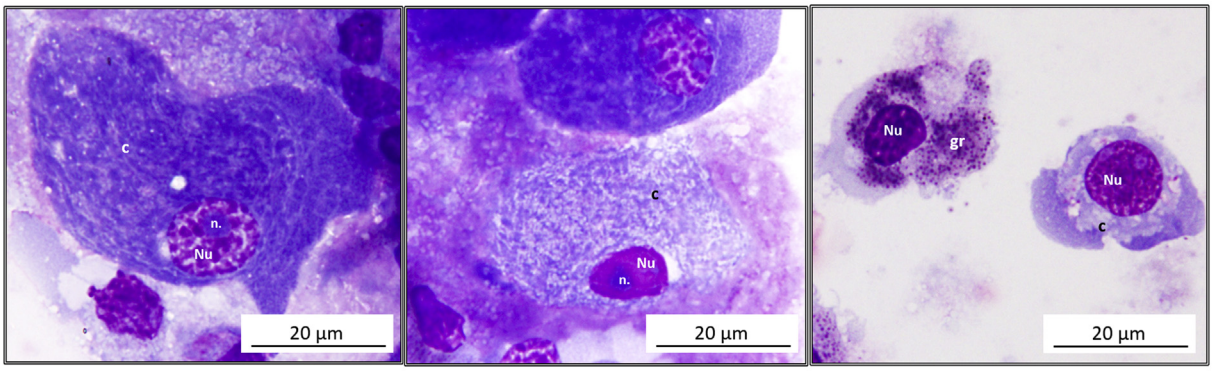

Fig. 2. Haemocyte cells, stained with Diff-Quick solution: (Nu) nucleus; (n.) nucleoli; (c) cytoplasm; (gr) granules.

exposure and a significant interaction between pH and LNG was observed (LM model, $F_{(3,111)} = 3.003$, $p < 0.05$) (Fig. 3, Table S1 and S1.1 in supplementary material), attributed to just one significant difference found for the solvent control at pH 8.1, presenting higher DNA damage than the remaining treatments.

To assess the DNA damage distribution, data were organized by classes according to the % of DNA in tail (Table 1), as described in Material and Methods section. For all the treatments, the most representative damaged classes were class 0 and 1 followed by class 4. Except for the treatment 22 °C, pH 8.1, SC that presented higher frequency for classes 2 and 4. Significant differences ($p < 0.05$) among treatments were found for classes 0, 2 and 3, as indicated in Table 1 (and Tables S2.1-S2.5 in supplementary material).

Considering that no differences were observed for the LNG treatments among the distinct temperature x pH combinations, a general analysis by hormone was done (Fig. 4 and Tables S3.1-S3.5 in supplementary material). For each class was applied a LM model and no differences were observed between LNG treatments and the negative control. Classes 0, 1 and 4 were the most prevalent in all treatments. However, the solvent control revealed to be significantly different ($p < 0.05$) from the other treatments, a significant decrease in class 0, while a significant increase in classes 2 and 4 was observed. These results followed the same pattern observed previously in Fig. 3.

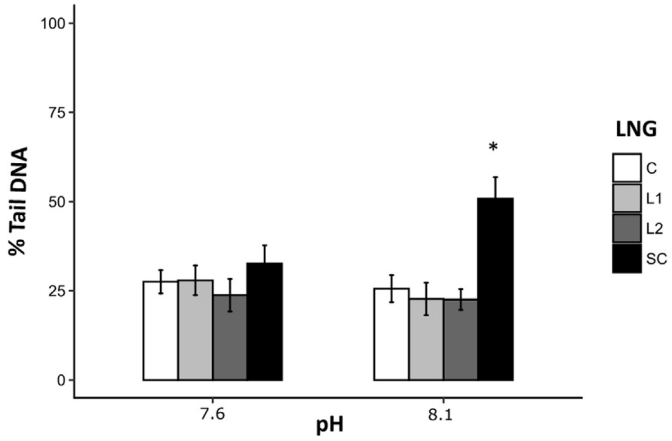

Fig. 3. DNA damage in haemocytes of *G. locusta* exposed to different treatments, expressed as average % Tail DNA (±SE; n = 6 replicates per treatment). Data is displayed relative to significant factors. *indicate significant differences among treatments (Tukey test, $p < 0.05$). C – control; L1 – LNG 10 ng L^{-1}; L2 – LNG 1000 ng L^{-1}; SC – solvent control.

3.4. Protective effects against H_2O_2-induced DNA damage

To assess the potential protective effects against H_2O_2-induced DNA damage, *ex vivo* haemocytes were exposed to H_2O_2 and strand breaks detected by comet assay. In general, DNA damages observed were higher than in the absence of H_2O_2 and a significant effect ($p < 0.05$) of the hormone was detected. Thus, the solvent control significantly showed higher DNA damages than the other treatments (LM model, $F_{(3,105)} = 11.57$, $p < 0.05$) (Fig. 5A and Tables S4 and S4.1 in supplementary material). Stress factors like temperature and pCO_2 did not interfere with the ability of H_2O_2 to induce DNA damage (mainly SBs) on the haemocytes of *G. locusta*, after twenty-one days of exposure. However, the factor hormone seems to increase the ability of H_2O_2-induced DNA damage in haemocytes (Fig. 5B) increasing the cumulative frequency of nucleoids in class 4 from 151 in the control to 193 and 179 for L1 and L2, respectively (raw data in Table 2).

Regarding the frequency of nucleoids for each class (Table 2 and Tables S5.1-S5.5 in supplementary material), it increased in class 3 and 4 in all the treatments when compared with the frequency of nucleoids in haemocytes without H_2O_2 exposure (Table 1). Considering a general analysis by hormone (Fig. 6 and Tables S6.1-S6.5 in supplementary material) for all classes, except class 0, there

Table 1
Mean frequencies (%) of nucleoids in each class (mean ± SE) measured by the comet assay, in haemocytes of *G. locusta* exposed to distinct treatments. n = 6 replicates per treatment. Significant differences are represented by symbols, where treatment marked with (•) is different from treatments marked with ($) inside of the same class, (Tukey test, $p < 0.05$). C – control; L1 – LNG 10 ng L^{-1}; L2 – LNG 1000 ng L^{-1}; SC – solvent control; nda: no data available.

	Class 0	Class 1	Class 2	Class 3	Class 4
18 7.6 C	38.4 ± 7.5	20.4 ± 3.4	9.9 ± 2.4	6.8 ± 1.8	24.5 ± 6.2
18 7.6 L1	41.6 ± 8.1	22.1 ± 3.8	6.0 ± 2.0$	7.7 ± 2.7	22.5 ± 7.1
18 7.6 L2	50.2 ± 8.6$	23.2 ± 4.3	8.0 ± 1.8$	4.2 ± 1.7$	14.4 ± 4.8
18 7.6 SC	11.0 ± 2.5•	37.1 ± 5.0	14.2 ± 1.4	7.9 ± 1.6	29.8 ± 5.2
18 8.1 C	47.4 ± 4.3	26.7 ± 4.2	5.9 ± 1.1$	3.5 ± 1.3$	16.5 ± 5.1
18 8.1 L1	57.0 ± 8.4$	17.2 ± 3.1	2.6 ± 1.3$	6.9 ± 2.7	16.3 ± 6.4
18 8.1 L2	44.5 ± 3.6	28.6 ± 4.0	6.3 ± 1.3$	6.1 ± 1.2	14.5 ± 2.7
18 8.1 SC	10.4 ± 4.4	33.4 ± 7.1	10.9 ± 3.1	6.6 ± 2.0	38.6 ± 9.1
22 7.6 C	39.5 ± 5.8	28.1 ± 5.7	6.4 ± 1.8$	3.3 ± 0.9$	22.8 ± 5.6
22 7.6 L1	37.1 ± 6.1	29.1 ± 5.7	9.9 ± 2.2	7.3 ± 1.7	16.7 ± 3.2
22 7.6 L2	40.7 ± 12.4	26.0 ± 4.8	4.2 ± 1.7$	5.5 ± 1.4	23.7 ± 9.1
22 7.6 SC	63.2 ± 13.2	35.3 ± 13.6	nda	0.5 ± 0.5	1.0 ± 0.1
22 8.1 C	28.8 ± 7.3	31.8 ± 4.1	10.4 ± 2.8	7.7 ± 2.7	21.3 ± 6.9
22 8.1 L1	50.7 ± 7.7$	21.8 ± 3.8	4.6 ± 1.2$	6.7 ± 1.6	16.1 ± 5.9
22 8.1 L2	47.5 ± 11.1$	23.1 ± 6.9	7.4 ± 2.8$	2.7 ± 0.8$	19.2 ± 7.7
22 8.1 SC	2.0 ± 1.3	17.3 ± 15.9	24.2 ± 9.2•	18.0 ± 3.7•	38.5 ± 10.1

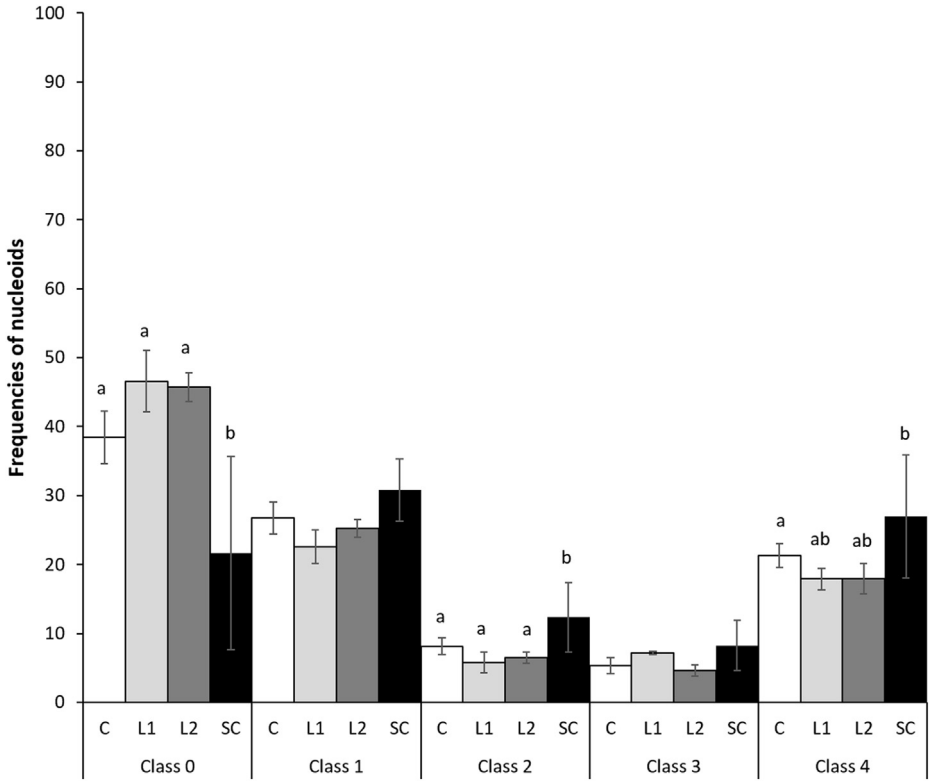

Fig. 4. Mean frequencies (%) of nucleoids in each class (mean ± SE) measured by comet assay, in haemocytes of *G. locusta* exposed to different LNG concentrations (n = 6 replicates per treatment). Different letters represent significant differences among treatments for each class, ($p < 0.05$). C – control; L1 – LNG 10 ng L^{-1}; L2 – LNG 1000 ng L^{-1}; SC – solvent control.

were significant differences between the solvent control and the other treatments (LM model, $p < 0.05$). For the solvent control the frequency of nucleoids decreased on classes 1, 2 and 3 and increased on class 4 where the % of nucleoids frequency was around 85%.

4. Discussion

The haemocytes play an important role in the immunological response in invertebrates, affecting their survival capability (Calisi et al., 2008; Ellis et al., 2011). So, evaluation of DNA damage in haemocytes may be a potential biomarker to access the impact of environmental factors on the organism immunological fitness and consequent survival. The present study assessed, for the first time, genotoxic effects of combined multiple stressors (temperature, pCO_2 and LNG concentrations), as well as, its potential effect on protection against H_2O_2-induced DNA damage on haemocytes of *Gammarus locusta*. The comet assay technique was successfully carried out to evaluate DNA damage using haemocytes collected from individual organisms. So far, few studies were done with crustaceans approaching genotoxicity; we can find some done with marine species (as the grass shrimp and the blue crab (Davolos et al., 2015; Lee et al., 2000, 1999)) and with the freshwater model *Daphnia* spp. (Lee et al., 2009; Park and Choi, 2009; Parrella et al., 2015). *Gammarus* sp. is an important sentinel model tolerant to a wide range of salinities and a crucial component in the benthic species food chain (Hou and Sket, 2016). Comet assay studies, done by Lacaze et al. (2011a, 2011c, 2010) and Di Donato et al. (2016), have already shown the importance and versatility of this model: *in vivo* and *in vitro* studies demonstrate the capability of different type of cells to respond positively to some chemicals, like cadmium chloride, mercury, 1,2-benzpyrene, paraquat, potassium dichromate and methyl methanesulfonate.

According to our findings, the cells observed in Fig. 2 are representative of those analysed through Comet assay, which are similar to the different haemocytes observed by Steele and MacPherson (1981). Moreover, none of the stress factors alone significantly increased SBs, detected by comet assay on haemocytes after twenty-one days exposure. However, a significant interaction between pH and LNG was observed showing a significant increase ($p < 0.05$) of % of DNA in tail in solvent control when compared to the control and other treatments (factors: pH and LNG). The analysis of the frequency of nucleoids by classes offers detailed information about the distribution of the undamaged and damaged nucleoids (Guilherme et al., 2010). This analysis, revealed that no differences were observed between the different treatments in the same class, except for the solvent control where it was observed a significant decrease ($p < 0.05$) of nucleoids frequency in class 0, while in class 2 and 4 was observed a significant increase ($p < 0.05$) relative to other treatments. The general analysis by hormone showed that around 20, 30 and 25% of the nucleoids from solvent control were in class 0, 1 and 4, respectively, while in LNG treatment, around of 45, 25 and 20% of nucleoids were in class 0, 1 and 4, respectively. These results suggest a genotoxic effect of solvent. In our recent published data, it was observed lower growth rates ($p < 0.05$) for animals exposed to ethanol (solvent control; Cardoso et al., 2018). This effect was hypothetically explained as an indirect mechanism resulting from solvent-stimulation of microbial growth that would decrease the dissolved oxygen and cause growth suppression (Cardoso et al., 2018; Hutchinson et al., 2006). Now, we showed that the solvent can also contribute to the observed DNA damage, probably by an increase of reactive oxygen species (ROS)

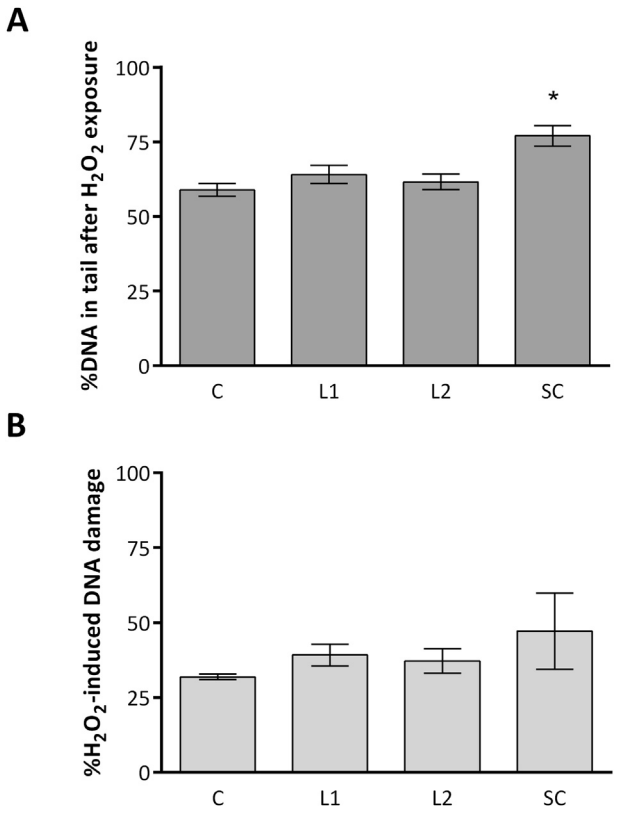

Fig. 5. DNA damage in haemocytes of *G. locusta* exposed to different treatments after *ex vivo* incubation with H_2O_2, expressed as average (±SE; n = 6 replicates per treatment). Data are displayed relative to significant factors. *indicates significant differences among treatments (Tukey test, $p < 0.05$). A) %DNA in tail after H_2O_2 exposure. B) Fraction of %DNA damage induced by H_2O_2 (DNA damage after H_2O_2 exposure − Basal DNA damage). C − control; L1 − LNG 10 ng L^{-1}; L2 − LNG 1000 ng L^{-1}; SC − solvent control.

due to a microbial growth. The increase of DNA damage, in solvent control, could partially explain the growth suppression observed before in *G. locusta*, since one of the consequences of DNA damage induction could be cell cycle arrest (Hoeijmakers, 2001). However, the "solvent effect" was not observed in the hormone treatments probably due to a "protective effect" of hormone that was also observed in the previous study (Cardoso et al., 2018). The underlying mechanism for such protection is unclear, namely in view that progestins may be genotoxic (Siddique and Afzal, 2008). Because the hormones impact is known on the microbiota (Neuman et al., 2015), one possible mechanism would be an action of LNG over microbial growth, contrary to the solvent. Once more, it is important to remember that no cytotoxicity was observed, since cell viability was superior to 80%, proving that genotoxicity data were not influenced by cytotoxic effects.

Besides all the advantages of comet assay, the variability of the results is an important issue mainly in *in vivo* assays due to the variability existent between animals — since each individual has a unique physiological response — increase the heterogeneity of results, and consequently increase the standard deviations, in the treatments. Other sources of variability such as slide-to-slide, cell-to-cell, electrophoresis conditions and comet analysis may impact the variability of the results (Hartmann et al., 2003). In this study several precautions, as referred by Hartmann et al. (2003), were taken to minimize the experimental variations, even the same individual doing the collection of haemolymph and analysis of the Comet assay data. However, in the control, the % of DNA in tail showed higher values (≈22–29%) than those obtained by Lacaze et al. (2011a, 2010), for the same type of cells isolated from *Gammarus fossarum*. This may suggest differences between species or different impacts according to husbandry conditions. However, in this case, physico-chemical parameters, like ammonia and nitrites, averaged normal amounts for a maintenance system and water quality was assessed before its use to avoid any extra stress to this model and affect the %DNA basal damage.

Toxicological studies, performed in different type of animals, proved already the fitness of haemocytes for genotoxic detection (Davolos et al., 2015; Klobučar et al., 2003; Lacaze et al., 2011a, 2010; Wang et al., 2016). However, the absence of increase of SBs by the stress factors (temperature, pH and LNG) in this study could be partially due to the extended experimental duration (twenty-one days), which could have allowed the adaptation of animals to the new conditions, repair of SBs and regenerate all their immunological cells in the meantime (Ellis et al., 2011). According to the previous results (Cardoso et al., 2018), it would be expected that organisms from the warming condition (22 °C pH 8.1), that caused higher lethal effects, would also present a higher percentage of DNA damage. However, this was not observed in the present work. The mammalian response to SBs repair involve several DNA repair enzymes that according to Kochan et al. (2017) are recruited to the place of the damage within a short time (within 15–20 min) repairing the damages, when possible. Nonetheless, in molluscs and crustaceans few information about the mechanisms of DNA damage and repair is available being necessary further studies about these basal mechanisms (de Lapuente et al., 2015) to better understand the results obtained. Previous studies from Lacaze et al. (2010) and Weber et al. (2003) in amphipods have revealed positive genotoxic effects of contaminants, under few days of exposure (i.e. 5 and 3 days, respectively). In our point of view, to clarify the genotoxic effect of these stress factors in *G. locusta*, different DNA damage (using DNA lesion-specific repair enzymes) at short period of exposure, such as few hours, should be evaluated in future studies.

Climate change may modify the sensitivity and susceptibility of organisms to chemicals while, on the other hand, the pollutants can damage the capacity of organisms to respond to rapidly changing climate conditions (Hooper et al., 2013; Noyes and Lema, 2015). To assess the effect of stress factors (temperature, pCO_2 and LNG) in

Table 2
Mean frequencies of nucleoids in each class (mean ± SE) measured by the comet assay after *ex vivo* incubation with H_2O_2, in haemocytes of *G. locusta* exposed to distinct treatments. n = 6 replicates per treatment. Significant differences are represented by symbols, where treatment marked with (•) is different from treatments marked with ($) inside of the same class, (Tukey test, $p < 0.05$). Besides, for class 3 there are significant differences between both pH levels, herein marked with A and B. C − control; L1 − LNG 10 ng L^{-1}; L2 − LNG 1000 ng L^{-1}; SC − solvent control; nda: no data available.

	Class 0	Class 1	Class 2	Class 3	Class 4
18 7.6 C	0.9 ± 0.5	4.9 ± 1.9$	21.5 ± 3.8$	36.2 ± 3.7$,A	36.5 ± 5.6$
18 7.6 L1	2.0 ± 0.7	10.0 ± 3.2$	19.0 ± 4.6$	24.7 ± 2.6$,A	44.3 ± 9.2$
18 7.6 L2	2.0 ± 0.6	6.4 ± 2.3$	14.2 ± 3.4$	24.9 ± 5.5$,A	52.5 ± 8.1$
18 7.6 SC	nda	0.7 ± 0.7•	3.4 ± 3.4•	10.9 ± 3.0•,A	85.0 ± 6.2•
18 8.1 C	3.8 ± 3.0	14.2 ± 4.7$	17.6 ± 3.1$	34.1 ± 4.7$,B	30.2 ± 5.5$
18 8.1 L1	0.4 ± 0.2	1.9 ± 0.6$	7.5 ± 1.7$	32.5 ± 4.2$,B	57.6 ± 5.2$
18 8.1 L2	0.6 ± 0.3	5.3 ± 1.3$	14.7 ± 3.4$	33.5 ± 2.2$,B	45.9 ± 5.1$
18 8.1 SC	0.2 ± 0.2	nda	0.3 ± 0.2•	14.7 ± 4.6•,B	84.9 ± 4.5•
22 7.6 C	1.3 ± 0.4	7.6 ± 2.6$	22.2 ± 3.9$	26.7 ± 3.5$,A	42.0 ± 5.2$
22 7.6 L1	3.1 ± 1.6	7.9 ± 5.0$	13.5 ± 3.6$	27.5 ± 3.8$,A	48.1 ± 9.2$
22 7.6 L2	3.5 ± 1.5	14.8 ± 6.3$	19.9 ± 3.9$	21.3 ± 4.9$,A	40.5 ± 11.4$
22 7.6 SC	nda	nda	0.4 ± 0.4•	8.6 ± 2.2•,A	90.9 ± 2.6•
22 8.1 C	1.1 ± 1.0	5.5 ± 2.5$	13.3 ± 4.0$	37.3 ± 5.2$,B	42.7 ± 9.7$
22 8.1 L1	1.2 ± 0.6	7.8 ± 2.3$	16.6 ± 3.4$	30.7 ± 3.8$,B	43.6 ± 8.4$
22 8.1 L2	3.5 ± 1.8	11.0 ± 4.8$	16.5 ± 4.3$	28.5 ± 4.8$,B	40.4 ± 9.7$
22 8.1 SC	nda	nda	1.0 ± 0.6•	22.3 ± 7.2•,B	76.7 ± 7.5•

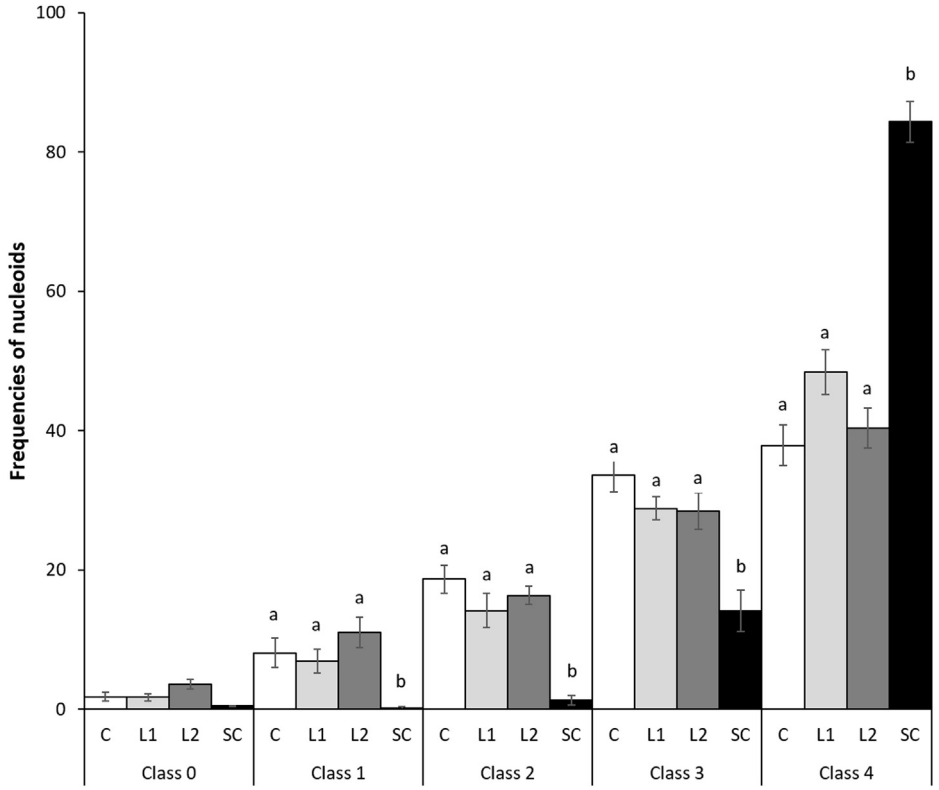

Fig. 6. Mean frequencies of nucleoids in each class (mean ± SE) measured by the comet assay after *ex vivo* incubation with H_2O_2, in haemocytes of *G. locusta* exposed to different LNG concentrations. n = 6 replicates per treatment. Different letters represent significant differences among treatments, ($p < 0.05$). C – control; L1 – LNG 10 ng L^{-1}; L2 – LNG 1000 ng L^{-1}; SC – solvent control.

the ability of haemocytes to respond to another stress factor, haemocytes of each treatment were exposed, *ex vivo*, to an oxidant agent – H_2O_2. Using the Comet assay, was observed and increase of DNA damage in all treatments after H_2O_2 exposure compared to basal damage, with class 4 being the one with the highest nucleoids frequency in all treatments. However, no significant differences were found relative to the control, after H_2O_2 exposure, except solvent control, where the nucleoids frequency in class 4 significantly increased compared with the other treatments. Regarding the effect of the hormone and the cumulative frequency of nucleoids, an increased tendency of the haemocytes ability of H_2O_2-induced DNA damage was observed, mainly in class 4 relative to the control. This means that the haemocytes in these conditions seem to be more susceptible to DNA damage induction than the haemocytes from the control. Finco et al. (2011) showed that estrogen/progestagen-combined oral contraceptive increased oxidative stress by depletion of antioxidant defences, namely the glutathione levels in humans. Reduction of antioxidant defences increase cell's susceptibility to a second oxidant insult (Birben et al., 2012); all this information is clearly missing in amphipods.

5. Conclusions

Our results indicate that, under the test conditions, none of the stress factors (temperature, pCO_2 and LNG) induced DNA damage (mainly SBs) or protected against H_2O_2-induced DNA damage in haemocytes isolated from *Gammarus locusta*, after twenty-one days exposure. However, LNG seemed to induce a slight increase of DNA damage after H_2O_2 exposure. An interesting data was the "solvent effect" that increased DNA damage in haemocytes; however this effect was not observed in hormone treatments suggesting a "protective effect" of hormone. Further studies should be performed to confirm these effects and explore the possible mechanisms involved. This study reinforces the relevance of the comet assay in ecotoxicology and risk assessment along with combined effects of multiple stressors. In addition, our results suggest that more short-term studies should be performed to conclude about the genotoxicity and/or protective effects of the stress factors in *Gammarus locusta,* attending to the fast turnover rate of repairing cells. In fact, a medium-term sampling could have been the main responsible for the lack of positive results at DNA level.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.11.070.

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